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A synthetic biology approach to the construction of membrane proteins in semi-synthetic minimal cells

Yutetsu Kuruma^a, Pasquale Stano^{a,b}, Takuya Ueda^c, Pier Luigi Luisi^{b,*}^a "Enrico Fermi" Study and Research Center, Via Panisperna 89A, 00184 Rome, Italy^b Department of Biology, University of Rome "RomaTre", Viale G. Marconi 446, 00146 Rome, Italy^c Department of Medical Genome Sciences, Graduate School of Frontier Sciences, The University of Tokyo, Kashiwanoha 5-1-5, Kashiwa-shi, Chiba, Japan

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ABSTRACT

Synthetic biology is an emerging field that aims at constructing artificial biological systems by combining engineering and molecular biology approaches. One of the most ambitious research line concerns the so-called semi-synthetic minimal cells, which are liposome-based system capable of synthesizing the lipids within the liposome surface. This goal can be reached by reconstituting membrane proteins within liposomes and allow them to synthesize lipids. This approach, that can be defined as biochemical, was already reported by us (Schmidli et al. *J. Am. Chem. Soc.* 113, 8127–8130, 1991). In more advanced models, however, a full reconstruction of the biochemical pathway requires (1) the synthesis of functional membrane enzymes inside liposomes, and (2) the local synthesis of lipids as catalyzed by the in situ synthesized enzymes. Here we show the synthesis and the activity – inside liposomes – of two membrane proteins involved in phospholipids biosynthesis pathway. The proteins, *sn*-glycerol-3-phosphate acyltransferase (GPAT) and lysophosphatidic acid acyltransferase (LPAAT), have been synthesized by using a totally reconstructed cell-free system (PURE system) encapsulated in liposomes. The activities of internally synthesized GPAT and LPAAT were confirmed by detecting the produced lysophosphatidic acid and phosphatidic acid, respectively. Through this procedure, we have implemented the first phase of a design aimed at synthesizing phospholipid membrane from liposome within from within – which corresponds to the autopoietic growth mechanism.

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1. Introduction

A new research avenue of modern synthetic biology concerns the construction of the minimal cell, i.e., a semi-synthetic cell containing the minimal and sufficient number of components to fulfil the basic properties of cellular life; i.e., self-maintenance, self-reproduction, and evolution [1]. This idea, which is also related to the concept of "minimal genome" [2], has been approached by different groups [1,3–5], and has reached a stage where proteins can be synthesized inside liposomes by using encapsulated cell-free translation system. What is missing until now is self-reproduction, which is actually the most characteristic and dynamic event of the living cell. A synthetic full reconstruction of cell division is still far from reality, and must be approached in subsequent steps of growing complexity. One first step is the self-reproduction of the external membrane. It has been already shown that liposomes can autopoietically grow and divide by external feeding with membrane precursors [6–8]. In addition, a preliminary attempt to the biosynthesis of phospholipids from the inside, based on the entrapment of four enzymes, has been also described, which

however did not appear as an efficient way to proceed [9]. A more advanced scheme would imply the expression of membrano-genic enzymes inside liposomes, and this is in fact the aim of the present paper. Here we have designed and built a synthetic cell that consists of a totally reconstructed cell-free translation system (PURE system) [10] encapsulated inside a liposome of proper lipid composition. The main goal of this work is the expression, inside liposomes, of two enzymes involved in the biosynthesis of phospholipid [11] (Fig. 1a), and in particular of phosphatidic acid (PA) starting from suitable precursors, i.e., *sn*-glycerol-3-phosphate and acyl-CoA (Fig. 1b). Both enzymes, *sn*-glycerol-3-phosphate acyltransferase (GPAT, EC. 2.3.1.15) and lysophosphatidic acid acyltransferase (LPAAT, EC. 2.3.1.51) are membrane proteins. The basic idea of the present work is thus the internal production of PA, which would translocate into the lipidic membrane, giving rise to liposome growth and eventually division. It has been in fact amply documented in our group that addition of fresh lipidic component into the membrane leads to vesicle growth and division [7,12]. Although some researchers have addressed the internal protein synthesis of liposome [13–15], they have been dealing generally with cytoplasmic water-soluble proteins. In our latest report [16], we have shown seminal data on the synthesis of membrane proteins inside liposomes. We show here, for the first time, that

* Corresponding author. Tel.: +39 06 5517 6329; fax: +39 06 5517 6321.

E-mail address: luisi@mat.ethz.ch (P.L. Luisi).

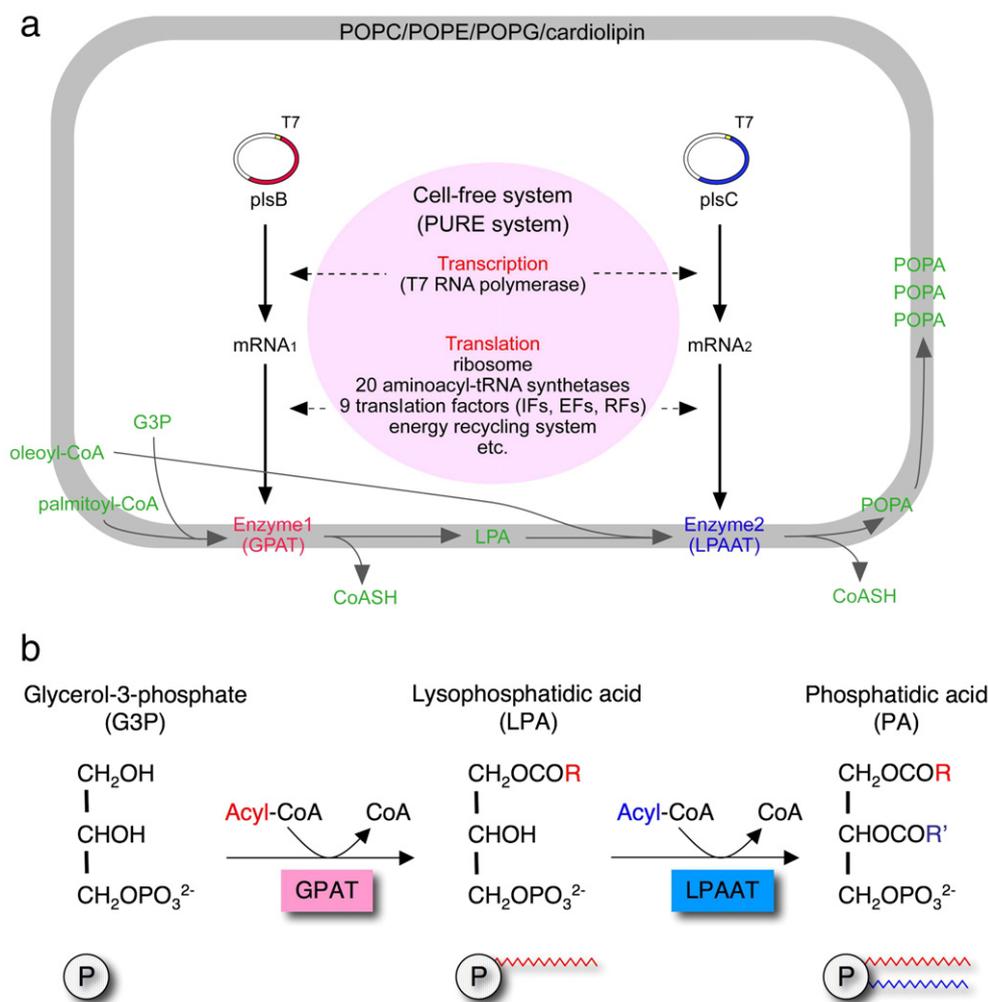


Fig. 1. Graphic of a lipid-synthesizing liposome. (a) All translational factors are encapsulated inside liposome, which is composed by four kinds of phospholipid. Two membrane proteins, GPAT and LPAAT, involved in phosphatidic acid biosynthesis pathway are expressed inside liposomes. Depending on the activities of the GPAT and LPAAT, phosphatidic acids are stepwise synthesized from glycerol-3-phosphate via lysophosphatidic acid. The resulting lipid is taken part in the composition of the own liposome that stimulates the increase of liposome surface for the self-reproduction. (b) Glycerol-3-phosphate (G3P) is first converted into LPA by GPAT. Then the resulting LPA will be a substrate for POPA, which catalyzed by LPAAT. A type of acyl-CoA is applied as an acyl-chain donor in both reactions. In this study, R and R' correspond to palmitoyl and oleoyl chain, respectively.

membrane proteins can be functionally synthesized inside vesicles of proper lipid composition, and that such proteins can synthesize lipidic components at the aim of stimulating liposome growth and self-division. Aimed to the achievement of autopoietic self-reproduction, the presented results face the constructive challenge of synthetic cells.

2. Materials and methods

2.1. Materials

1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphatidylcholine (POPC), 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphatidylethanolamine (POPE), 1-palmitoyl-2-oleoyl-phosphatidylglycerol (POPG), 1-palmitoyl-2-oleoyl-phosphatidic acid (POPA), cardiolipin, *Escherichia coli* polar lipid extract, 1-palmitoyl-2-hydroxy-phosphatidic acid (LPA), oleoyl-CoA, and palmitoyl-CoA were purchased from Avanti Polar Lipids (Canada). *Sn*-glycerol-3-phosphate and L-Glutathione oxidized were obtained from Sigma-Aldrich. All radioisotope labeled materials, [^{14}C] *sn*-glycerol-3-phosphate, [^{35}S]methionine, and [^{14}C]oleoyl-CoA were purchased from HG Healthcare. A reconstructed cell-free translation system, PURE system, was provided from Post Genome Institute Co., Ltd. (Tokyo, Japan) or prepared in the original laboratory. The plasmids

pBT302_plsB and pBT302_plsC, containing the genes *plsB* and *plsC*, respectively, for the enzymes GPAT and LPAAT were supplied from BioTecton (Zurich). These genes are located under T7 promoter.

2.2. Liposome preparation

For 50 μL cell-free reaction mixture, 10 μmol of lipid(s) dissolved in chloroform was evaporated by centrifuge-evaporator within tube. For 150 μL cell-free mixture, 20 μmol of lipid(s) dissolved in chloroform was evaporated by rotary evaporator in 50 mL round-bottom flask. In both cases, chloroform was completely evaporated under very low pressure by pump for over night. Resulting lipid(s) films were combined with prepared cell-free reaction mixture and processed by bath sonication and vortexing to form liposomes.

2.3. Cell-free synthesis

All protein syntheses were carried out by the PURE system according to the procedure recommended by the manufacturer. For testing protein synthesis with radioisotope labelled amino acid, the mixtures were prepared in 25 μL and supplied with [^{35}S]methionine. For the detection of synthesized protein activity, the mixtures were prepared in 150 μL without radioisotope and mixed with lipid film in a

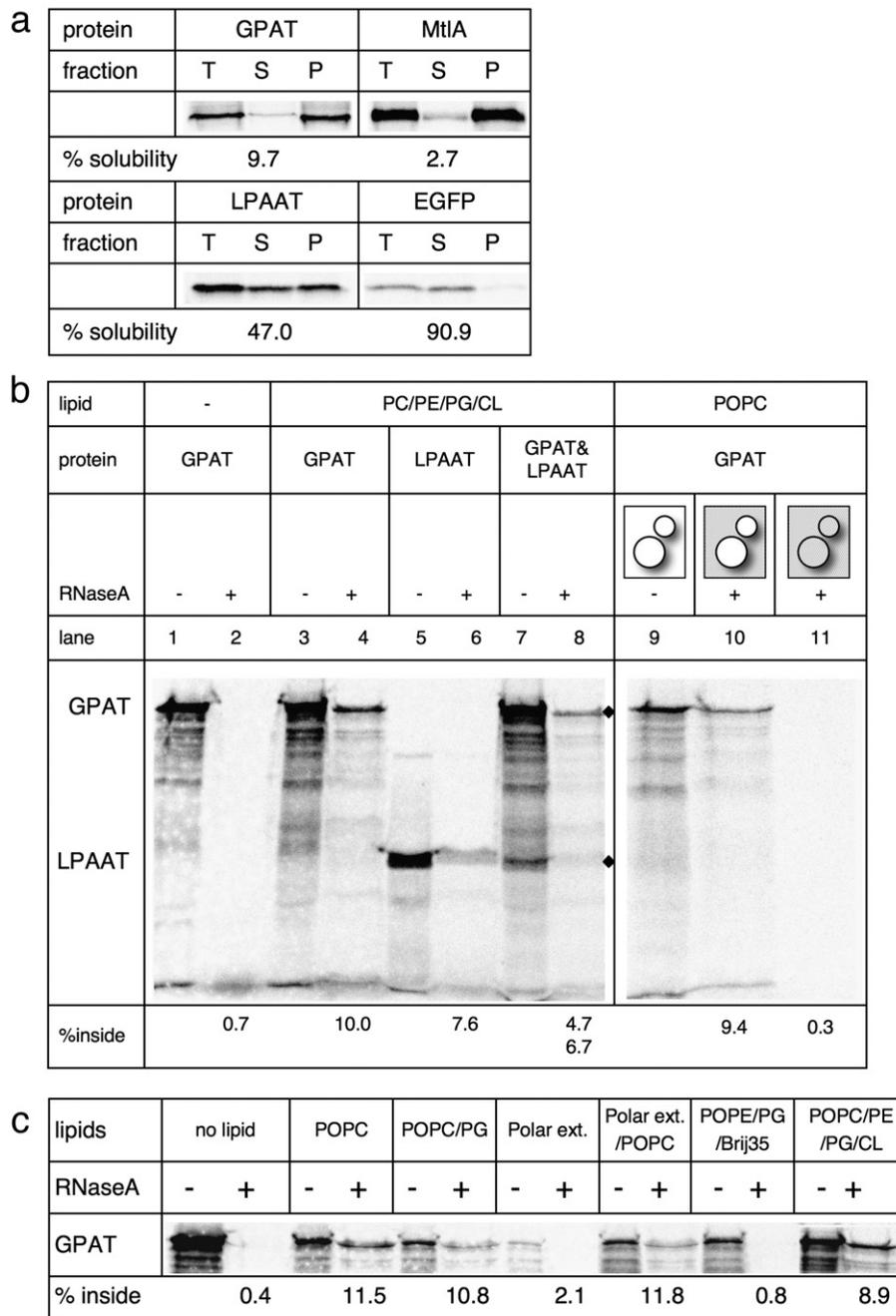


Fig. 2. Protein synthesis inside liposomes. (a) Solubility of GPAT and LPAAT. GPAT and LPAAT were synthesized in the PURE system and divided into soluble or insoluble fractions. Products were classified into total product (T), supernatant (S), and precipitate (P). A ratio of the soluble products is indicated at bottom of the gel. (b) Protein synthesis inside liposomes. GPAT and LPAAT were synthesized in the presence or absence of phospholipids. To prevent the outside synthesis, RNaseA was added after liposome forming (even lanes). On the lanes of RNaseA -, 20% of yields were loaded to the gel, whereas the whole of RNaseA+ samples were loaded. The positions of GPAT and LPAAT products are indicated on the column side, as diamonds. The percentages of inside synthesis are indicated at bottom of the gel. (c) Protein synthesis with several phospholipids. GPAT was synthesized in the presence of several lipid compositions with a set of RN - or +. The percents of inside synthesis are indicated at bottom of the gel. In all cases, proteins are synthesized in the presence of [³⁵S]methionine. The gels were developed by autoradiography and analyzed by MultiAnalyst (Bio-Rad).

flask, then the resulting mixtures were transferred in Eppendorf tube. In order to prevent the exterior synthesis, RNaseA was additionally added into the prepared mixture at 20 ng/ μ L (final conc.). The synthesis reactions were carried out at 37 °C for 4 h. For further analysis by SDS-PAGE gel, samples were processed by acetone precipitation twice and diethyl ether rinse once. The resulting precipitates were dried up and dissolved in loading buffer. The SDS-PAGE gel, 12% of acrylamide, was employed for all experiments. Then, the gel was dried and analyzed by autoradiography. The gel images were developed by Molecular Imager System GS-525 and analyzed by MultiAnalyst (Bio-Rad).

2.4. Measurements of the enzyme activities

After the protein synthesis, the sample mixture was diluted up to 500 μ L with appropriate buffer (GPAT buffer: 150 mM Tris-HCl (pH 8.4), 200 mM NaCl, 1 mg/mL bovine serum albumin, 5 mM β -mercaptoethanol, 5 mM MgCl₂; and LPAAT buffer: 100 mM Tris-HCl (pH 9.0), 1 mg/mL bovine serum albumin, 0.5 mM MgCl₂), and transferred into a small glass vial with micro magnetic stirrer bar. The mixture was stirred at 4 °C for over night and then the activity assay was performed as follows. For GPAT assay, the sample mixture (500 μ L) was supplied 100 μ M palmitoyl-CoA, 100 μ M G3P, and

10 μM [^{14}C]G3P in 1 mL volume, with GPAT buffer. The resulting mixture was processed five times by freeze-and-thaw in liquid nitrogen (F/T) to entrap the substrates into liposomes, and then incubated at 22 °C for 1–2 h. For the assay of liposomes inside, the concentration of G3P was decreased at 10 μM , and five F/T cycles were performed before incubation. For LPAAT assay, 50 μM LPA, 5 μM oleoyl-CoA and 5 μM [^{14}C]oleoyl-CoA were supplied in 1 mL mixture, with LPAAT buffer. The mixture was processed five times F/T and incubated at 22 °C for 1–2 h. For the assay of liposomes inside, the substrates were firstly added after the protein synthesis and entrapped by F/T. The mixture was combined with LPAAT buffer up to 1 mL, then proteinase K (proK) was added at 0.1 mg/mL (f.c.). The resulting mixture was transferred into the vial and stirred for 2 h at 4 °C, then incubated for 1 h at 22 °C. After the acyltransferase assays of GPAT and LPAAT, the produced LPA or POPA were extracted from the sample mixture according to the reports of Green et al. [17]. The chloroform fraction was collected and dried up for further analysis. The dried lipids were re-dissolved in 60 μL chloroform, and the half volume was used for liquid scintillation counter (LSC) measurement. A part of the remaining products were spotted on TLC Silica gel plate (Merck) and eluted with chloroform: methanol: 28% ammonia: H₂O (6.4: 3.0: 0.178: 0.422). The plate was analyzed by iodine vapour or autoradiography. GPAT and LPAAT semi-coupling assay was performed as follows. First, each protein was independently synthesized in 150 μL PURE system in the presence of PC/PE/PG/CL lipids, but without RNaseA, then the each 60 μL of reaction mixtures were mixed. The mixed mixture was combined with 500 μL with GL buffer (100 mM Tris-HCl (pH 8.4) and 100 mM NaCl). After the over night stirring at 4 °C, 20 mM [^{14}C]G3P, 100 mM palmitoyl-CoA, and 5 mM β -mercaptoethanol were introduced with GL buffer, up to 1 mL. Following 5 F/T cycles, the first enzyme reaction was performed for 1 h at 22 °C. For the subsequential second enzyme reaction, 5 mM oleoyl-CoA and 5 mM glutathione oxidized were additionally added, then mixture was processed 5 times F/T and incubated additional 1 h.

3. Results

3.1. Membrane protein synthesis inside liposomes

GPAT (83 kDa) [18] and LPAAT (27.5 kDa) [19] are synthesized by the PURE system in bulk water to verify their hydrophobic nature. The synthesized proteins were divided into the soluble or insoluble fractions. Almost 90% of synthesized GPAT is found in the precipitate fraction (Fig. 2a) and so is the case of maltose permease (MtlA), that is a typical integral membrane protein. LPAAT is instead found in the precipitate fraction and partially in the soluble one, as evidenced by the comparison with enhanced green fluorescence protein (EGFP), a typical soluble protein (Fig. 2a).

Following the solubility assessment, we performed protein synthesis inside liposomes by entrapping the PURE system and the corresponding template DNA(s) inside appropriate liposomes (mentioned below). The external reaction was inhibited by the addition of RNaseA after the liposome formation. As shown in Fig. 2b, GPAT and LPAAT were successfully produced in the presence of liposomes (lane 4, 6, 8, and 10). Liposomes are impermeable to macromolecules, which do not allow RNaseA digestion of internalized components. When both template DNAs (*plsB* and *plsC*, codifying for GPAT and LPAAT, respectively) were introduced, the simultaneous synthesis of GPAT and LPAAT was observed (lane 8). In order to verify that RNaseA is fully functional even in the presence of high phospholipids concentration, we performed a control experiment by adding RNaseA before the liposome formation. In this case, no protein synthesis was observed (lane 11). This result further confirms that GPAT and LPAAT are synthesized only inside liposomes when RNaseA is present outside liposomes.

Table 1
Phospholipid compositions of liposome

Lipid composition (mol)	Conc. (mM)	Synth.	Encap.	Act.
POPC	200	++	+++	–
POPC/POPG (80:20)	200	+	+	+
Polar lipid extract (<i>E. coli</i>)	1 mg/mL	±	–	+++
Polar lipid extract/POPC (50 ^a :50)	200	+	+	+
POPE/POPG/Brij35 (76.5:10.9:12.6)	50	+	–	Not tested
POPC/POPE/POPG/cardioliipin (50.8:35.6:11.5:2.1)	200	++	++	+++ ^b
No lipid	–	+++	–	–

Contributions of phospholipids compositions were compared on protein synthesis (Synth.) with GPAT protein, encapsulation (Encap.), and enzyme activity (Act.). The correlation between the each compositions are evaluated as (i) Synth.; a yield of PURE system (w/o lipid) is defined as a basis (100%), (ii) Encap.; a yield of protein synthesis inside POPC (200 mM) liposomes is defined as a basis, and (iii) Act.; the GPAT activity with *E. coli* polar lipid extract is defined as a basis. In all case, correlations are fixed as +++: 100% (basis), ++: >30%, +: 10–30%, ±: <10%, -: 0%.

^a The amount of lipid are converted into a molar.

^b The lipid concentration was employed at 133 mM.

Optimal lipid concentration for internal protein synthesis ranges between 100 and 200 mM (final concentration) (data not shown), where the PURE system synthesized almost 1 μg of protein inside liposomes in 1 mL. This value corresponds to 10% of yield in bulk PURE system. Thus, we carried out all following experiments with 200 mM lipids; or 133 mM for enzyme activity assay. Since phosphatidylcholine lipids alone do not provide a suitable membrane environment for the function of GPAT, several lipid compositions were investigated at the aim of achieving the internal synthesis and the acyltransferase activity (see Table 1). Based on the lipid composition of *E. coli* cytoplasmic membrane, three kinds of lipid – phosphatidylethanol, phosphatidylglycerol, and cardioliipin – were combined with POPC lipids at a certain ratio. As shown in the Fig. 2c, POPC/POPE/POPG/cardioliipin (50.8:35.6:11.5:2.1 (mol)) (PC/PE/PG/CL) liposomes effectively synthesized GPAT in their internal space, showing an effective encapsulation for the inside synthesis. Additionally, the PC/PE/PG/CL liposomes effectively contribute to the function of GPAT.

3.2. Acyltransferase activity of GPAT and LPAAT

As a first assay to prove acyltransferase activity, GPAT was synthesized in the PURE system and then the resulting product was mixed with *E. coli* polar lipid extract (PL). In this way, the expressed GPAT interacts with the lipid bilayer of the liposomes. Using the resulting proteoliposomes, the acyltransferase activity was assessed by adding [^{14}C]-glycerol-3-phosphate and palmitoyl-CoA, in order to form [^{14}C]-1-palmitoyl-*sn*-glycerol-3-phosphate (lysophosphatidic acid, LPA). The LPA product was quantified by scintillation counting and identified by thin layer chromatography (TLC). Significant counts due to produced LPA were detected only when synthesized GPAT was mixed with PL to form liposomes (Fig. 3a). Because the obtained counts were highly depended on PL lipids, the GPAT seems to be incorporated in liposome membrane. This demonstrates that GPAT cannot be active by itself and that a lipid matrix is required for its enzymatic activity. Produced LPA was further analyzed by TLC. The Rf value of LPA spot on TLC plate is ca. 0.13. Fig. 3a reveals that the LPA spot on the TLC plates were properly detected, whereas no relevant spots were obtained in the control experiment (expression of MtlA). Therefore, GPAT synthesized in the PURE system is enzymatically active after its association with phospholipids.

Next, GPAT was synthesized in the presence of PC/PE/PG/CL, and then tested for its enzymatic activity. When RNaseA was not added during protein synthesis, LPA was strongly detected on the TLC plate (Fig. 3b left). The estimated yield of LPA is about 2–3 nmol in 120 μL reaction mixture. This value shows that more than 10% of the added

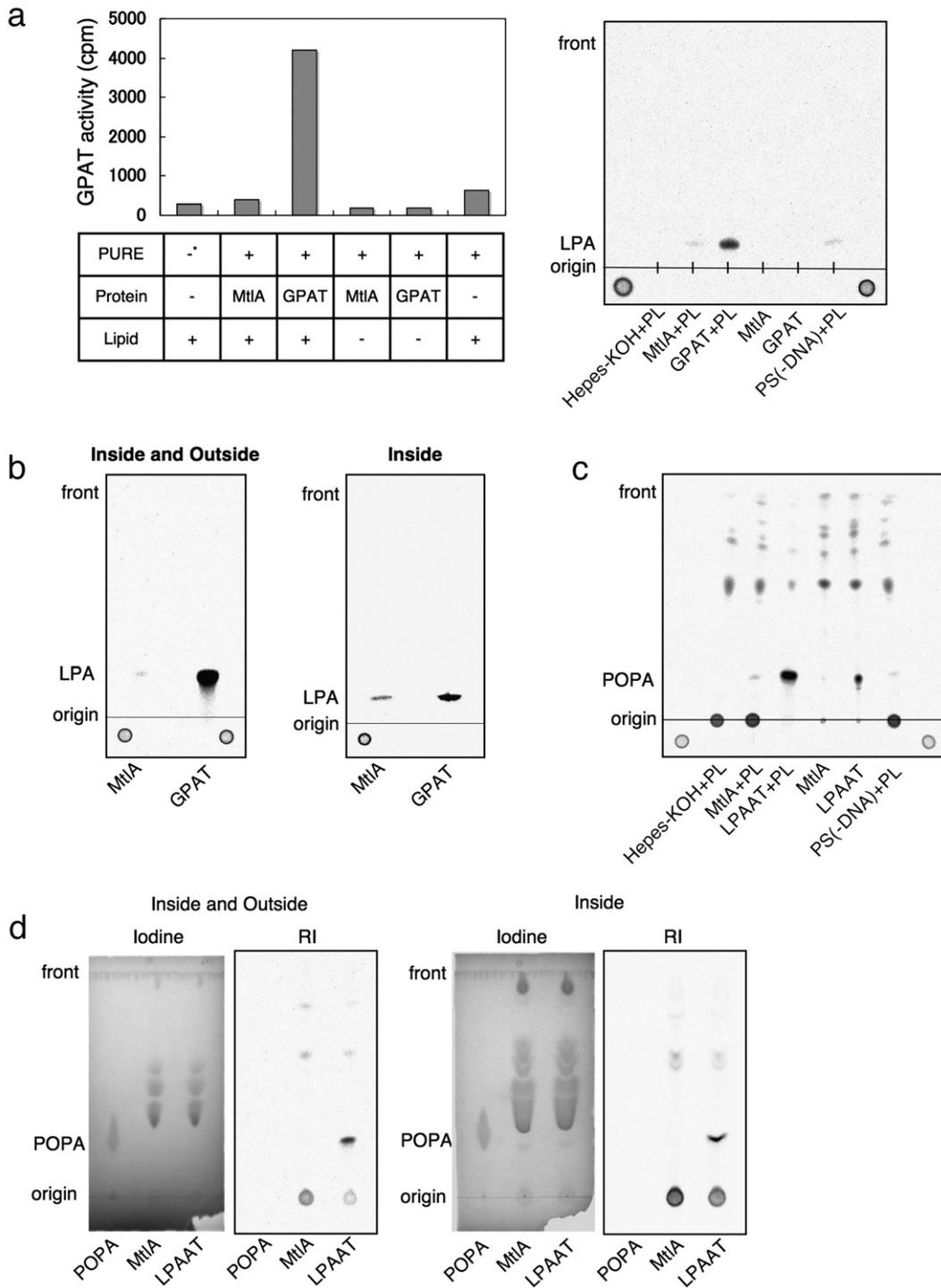


Fig. 3. Acyltransferase activities of the synthesized membrane proteins. (a) GPAT was synthesized in the PURE system and mixed with *E. coli* polar lipid extract. The acyltransferase activity was measured with a series of control in the presence of [¹⁴C]glycerol-3-phosphate, glycerol-3-phosphate, and palmitoyl-CoA. Same samples were also analyzed by thin layer chromatograph (TLC). (b) GPAT was synthesized in liposomes-coupling PURE system in the presence (left) or absence (right) of RNaseA. The resulting liposomes were analyzed for the GPAT activity with the set of substrates. (c) LPAAT was synthesized in the PURE system and mixed with *E. coli* polar lipid extract. The acyltransferase activity was measured with a described controls in the presence of [¹⁴C]oleoyl-CoA, oleoyl-CoA, and LPA. (d) LPAAT was synthesized in the presence of phospholipids as same as the GPAT case. In order to detect the liposomes inside activity, proteinaseK was added to digest the outside proteins. The resulting liposomes were tested in the LPAAT activity with the set of substrates. The positions of origin, front, and LPA or POPA product are indicated. The control lacking PURE system (*) was substituted by Hepes-KOH buffer. MtIA was used as a control of which does not have acyltransferase activity. The details of the experimental steps are described in [Materials and methods](#).

substrate was converted into LPA. When RNaseA was added to prevent the outside GPAT synthesis, the spot of labelled LPA, although weaker than in the previous case, was significantly detected on the TLC plate (Fig. 3b right). In this case, 40 pmol of LPA were enzymatically produced from GPAT, which in turn has been expressed inside

liposomes. This value indicates that more than 15% of entrapped substrates were converted into LPA. The so produced LPA, being poorly water-soluble, has to migrate into the lipidic membrane. In conclusion, all this means that we succeeded in producing an integral membrane protein inside liposomes and in detecting its enzymatic

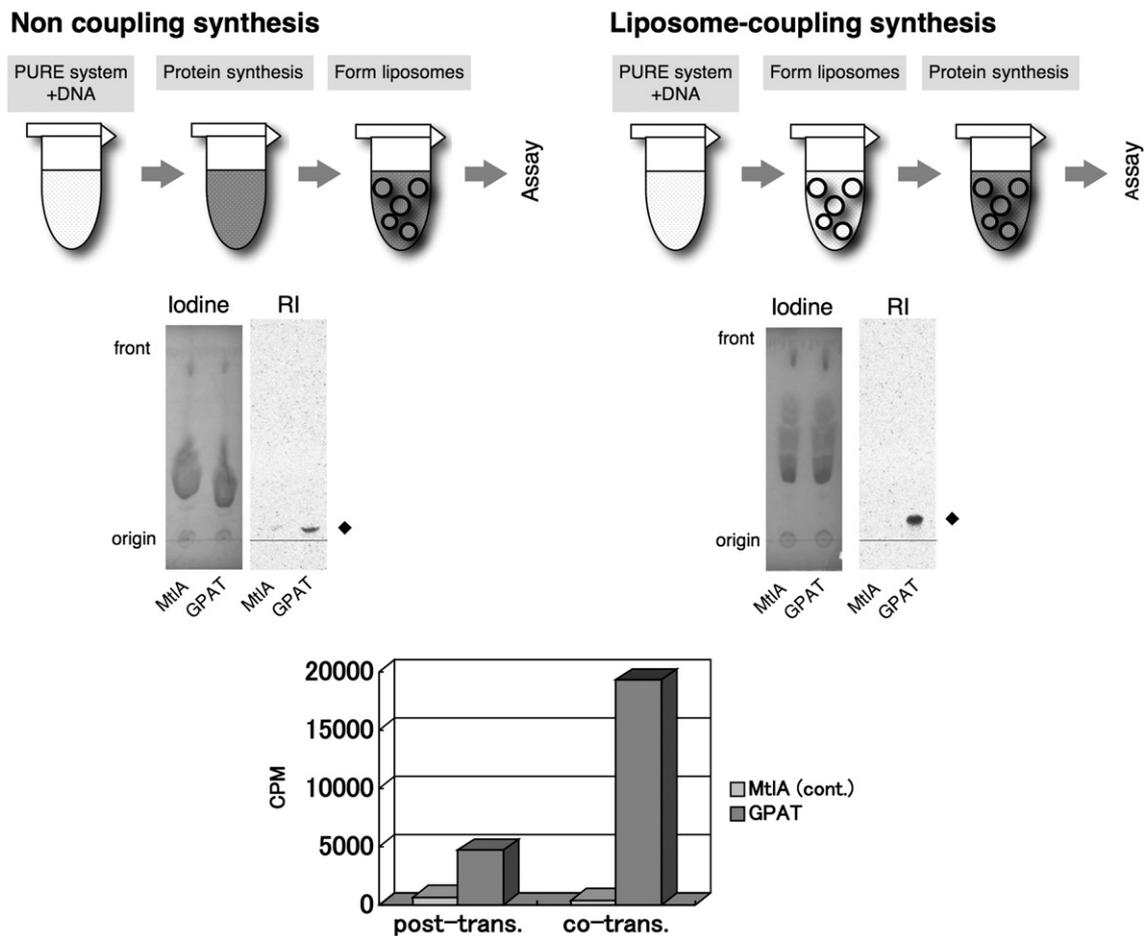


Fig. 4. Contribution of phospholipids for the membrane protein activity during the protein synthesis. GPAT was synthesized in the absence (non-coupling synthesis) or presence (liposomes-coupling synthesis) of phospholipids, and tested in its activity. A brief summary of procedure is described as figures. The LPA products were analyzed with TLC under iodine vapour and autoradiography. The position of LPA products is indicated as diamonds. The resulting spots are measured and compared in a graph with MtlA control.

activity for the production of membrane molecular components, both reactions occurring inside the liposomes.

LPAAT synthesis and activity were also demonstrated following the case of GPAT. The acyltransferase activity of synthesized LPAAT was first detected by mixing LPAAT (produced in the PURE system) with PL. After addition of LPA and [^{14}C]-oleoyl-CoA the resulting [^{14}C]-

palmitoyl-2-oleoyl-*sn*-glycerol-3-phosphatidic acid (POPA) was observed only when the synthesized LPAAT was combined with PL (Fig. 3c). Interestingly, a small amount of POPA (18%) was also detected in the absence of PL. As mentioned before, LPAAT was partially soluble in the absence of PL, which may result in this activity. POPA was also detected when LPAAT was produced inside and outside liposomes

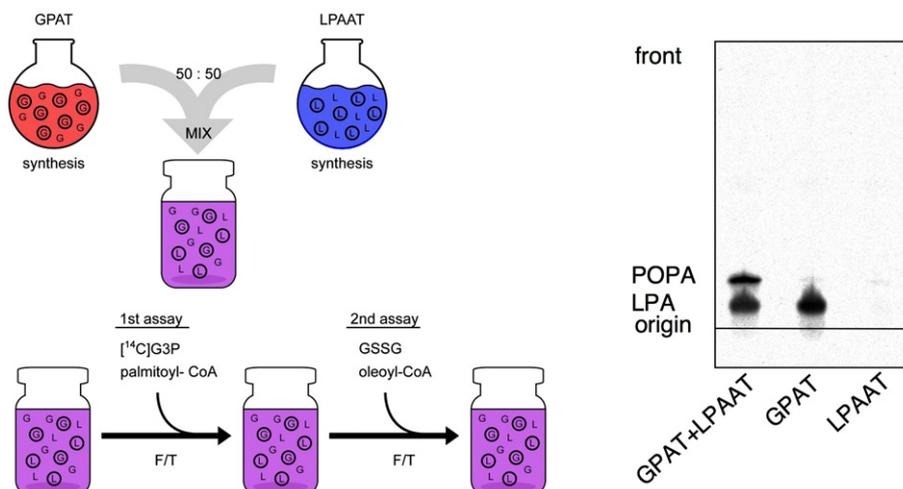


Fig. 5. Semi-coupling reactions of GPAT and LPAAT. GPAT and LPAAT were independently synthesized in liposomes-coupling PURE system, and both samples were mixed as 50:50 (%). Both acyltransferase assays were performed as stepwise as shown in figure. The positions of LPA and POPA products are indicated.

(Fig. 3d left). Whereas un-reacted oleoyl-CoA was not shifted from the origin (MtIA control), the POPA spot was observed at the proper position when compared with pure POPA (the Rf value POPA is ca. 0.25). The LPAAT activity only inside liposomes was also estimated by proteinaseK treatment (see [Materials and methods](#)), showing that POPA was successfully produced with a yield of almost 2 nmol.

3.3. Liposome-coupling translation contributes in membrane protein activity

We carried out additional experiments aimed to further understand the conditions for GPAT synthesis and activity. In particular, it was interesting to assess the contribution of liposome bilayer on the formation of functional GPAT. In these experiments, RNaseA was not introduced, in order to detect the strongest response in terms of LPA concentration. As shown in Fig. 4, when GPAT synthesis was carried out before liposome formation, the GPAT activity was about 25% lower than in the case of GPAT synthesis in the presence of liposomes, as judged by the LPA spot intensity on TLC plate. The result suggests that protein synthesis, which has taken place on ribosomes, favourably couples the peptide chain production with lipid bilayer interactions, which leads to functional membrane proteins. On the other hand, when GPAT is synthesized in the absence of a lipid matrix, synthesized (but aggregated) GPAT can still correctly interact with lipids, but only low activity was detected.

3.4. Semi-coupling of GPAT and LPAAT reactions

The final goal of this study is the simultaneous expression of active GPAT and LPAAT in a single liposome. This would allow the construction of a liposome, which builds its own membrane components. One problem lies in the coupling of the two enzymes, since the conditions for best enzymatic activity of the two enzymes differ. In particular, GPAT activity requires reductive conditions for its activity, whereas LPAAT requires an oxidative condition (data not shown). Based on these facts, we designed a two-step semi-coupled system as follows. First, each protein was independently synthesized in the presence of PC/PE/PG/CL lipids, but in the absence of RNaseA, then the two liposome populations were mixed (1:1 (v:v)). Second, GPAT activity was driven by adding G3P and palmitoyl-CoA to produce the LPA, which became the substrate of LPAAT. Then, after addition of oxidized glutathione (GSSG), the last substrate was added (oleoyl-CoA), and the products were analyzed by TLC (Fig. 5 left). By the addition of GSSG, the system is converted into an oxidized state that allows disulfide binding of cysteine residues. Both LPA and POPA spots were detected by using this semi-coupled system, whereas only LPA product was correctly observed when LPAAT was deliberately omitted (Fig. 5 right). When GPAT was not present, we did not observe any product. This evidence suggests that the two-step metabolic biosynthesis of phosphatidic acid may actually occur in a single mixture when its redox state is properly modulated.

4. Discussion

In this report, we have succeeded to produce two functional membrane proteins inside of liposomes by combing the cell-free translation system and liposome technology. The first enzyme, GPAT, is an integral membrane protein containing several transmembrane domains. The second enzyme, LPAAT, is a membrane anchoring protein supposed to be functional at periplasm region. This is the first evidence that an integral membrane protein can be synthesized inside liposomes, and its activity successfully detected. The presented liposomes are constructed by four kinds of phospholipid; i.e., POPC, POPE, POPG, and cardiolipin. This phospholipids composition does not interfere with the protein synthesis reaction, and, additionally, makes possible the activity of the integral membrane protein. Moreover, the

addition of oxidized glutathione activated the second enzyme. This fact suggests that disulfide bounds of the second protein were built post-translationally, by shifting the condition from reducing to oxidizing. Consequently, the protein was able to assume the functional conformation.

Although the activities of synthesized integral membrane proteins were detected inside liposomes, the low product yield and the forced discontinuity between the first and second reaction, however, do not allow yet direct observation of morphological changes in lipid-synthesizing liposomes. The amount of the phospholipids product is restricted by the fact of that liposomes do not allow permeability of water-soluble substrates. In order to overcome this problem, the liposomes must be a semi-open system as with living cell. One of the hints can be obtained from glycerol-3-phosphate (G3P) transporter in *E. coli* [20]. If the transporter is embedded on the liposome membrane in proper way, the substrate G3P substrate can be inputted continuously from outside. This transporter protein, however, contains 12 transmembrane regions [20]. Thus, some difficulty might be encountered in building such transporter on the liposome membrane. And as emphasized above, the other limit is that the second enzyme requires oxidative condition to be functional, whereas the first enzyme and cell-free protein synthesis reactions need reductive conditions. Concerning this problem, a possible solution might involve the use of a genetically engineered LPAAT that largely maintains the enzyme activity without disulfide bonds.

Although several related issues need deeper investigation (e.g., the actual interaction between expressed proteins and lipid matrix, as well as the development of a more effective strategy to coupling the activity of different enzymes inside vesicles), this study clearly sets another important milestone in the pathway towards the construction of self-reproducing minimal cells within the framework of synthetic biology.

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